AEROBES ASSOCIATED WITH THE METHANE FERMENTATION DURING FORMATE UTILIZATION

by

MARILYN EMILIE RINEHART

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Approved: Louis R Tsuia

Major Professor

TABLE OF CONTENTS

INTRODUCTION

The methane bacteria are ubiquitous in anaerobic environments in nature. Decaying vegetation in swamps and the black mud of lake beds produce bubbles of marsh gas or methane which have been observed for many centuries, especially in late summer when the water is warm and the microbial activity is high. More recently, methane bacteria have been found to be associated with other habitats as the sludge from sewage disposal plants and the digestive tracts of ruminants.

When cultivated in the laboratory, the methane bacteria are exceedingly sensitive to oxygen and, in handling them, one must observe strict precautions to insure anaerobiosis. Because of their strict anaerobic requirements, their relatively slow rate of growth, and their almost stubborn refusal to grow on solid media, they are difficult to purify and enrichment cultures[1] carried over months or years will contain one or more persistent contaminants.

Such a contaminant occurring in cultures producing methane has been observed repeatedly in this laboratory. It is of special interest, for when isolated, it shows a strict requirement for aerobic conditions. Apart from the methane-producing enrichment culture, it will grow only aerobically. In conjunction with the methane fermentation, it grows under what seems to be strict anaerobic conditions.

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[1] Enrichment cultures in this thesis refer to cultures in which the substrate and environmental conditions were chosen to favor development of species of methane bacteria which utilize a formate substrate without, however, inferring that the culture is pure as to both methane-producing and nonproducing types of bacteria.

This thesis represents an investigation of the characteristics of this peculiar "aerobe." The research involved divided itself naturally into three rather separate and distinct parts. First, the methane fermentation was characterized in tracer studies to determine that it was a typical and normal methane fermentation. Second, the organism to be studied was isolated and examined by conventional techniques, and third, a series of experiments were performed in an effort to elucidate the nature of the relationship between this organism and the strictly anaerobic methane fermentation.

REVIEW OF LITERATURE

That methane is characteristically produced by microbial decomposition of organic compounds in the absence of oxygen has been known for a long time. Pliny, in Roman times, described the escape of combustible gases from the earth's surface; Volta in 1776 recognized the relationship between the amount of combustible gas evolving from the bottom sediments of a lake and the amount of plant material in the sediment; Henry in 1806 showed that Volta's gas was the same as synthetic illuminating gas; and Bechamp in 1886 implicated a "living ferment" in the formation of methane from the decomposition of sugar and starch in the absence of oxygen (Barker 1956). It is only relatively recently that the metabolic pathways of the elusive methane bacteria have been elaborated and, indeed, the pathways have not yet been agreed upon by bacteriologists themselves. The bacteria have remained difficult to characterize because of the difficulty involved in isolating, growing, and maintaining pure cultures.

The Methane Bacteria

Before 1910 it was generally thought that methane was a direct result of the anaerobic decomposition of cellulose, the most abundant component of plants, but the bacteria responsible could not be isolated in pure culture. Then in 1910 Söhngen demonstrated that the products of cellulose fermentation, as formate, acetate, butyrate, ethanol, hydrogen, and carbon dioxide were more likely the substrates used by methane-producing bacteria, but his work was done using only a crude culture of methane bacteria. In fact, until 1936, all attempts to isolate pure cultures, or even to obtain growth of colonies in solid media, were unsuccessful (Barker, 1936).

Since 1936, four species of methane bacteria have been isolated in pure culture, Methanobacillus omelianskii (Barker, 1940), Methanobacterium formicicum (Schnellen, 1947), Methanosarcina barkerii (Schnellen, 1947), and Methanococcus vannielii (Stadtman and Barker, 1951). An additional four species have been isolated from cultures of other methane bacteria, but they have not been isolated from other anaerobic bacteria present in the culture. Because of the difficulties encountered in the isolation and maintenance of pure cultures of methane bacteria, physiological studies are generally carried out with highly enriched cultures rather than pure cultures.

All of the bacteria that produce methane are obligately anaerobic bacteria and develop only in the absence of oxygen and in the presence of a suitable reducing agent (Barker, 1936). The methane bacteria are much more sensitive to oxygen or other oxidizing agents, such as nitrate, than are most other anaerobic bacteria. For this reason they can be more easily grown in liquid or semisolid media. Solid sediments, as diatomaceous earth and shredded asbestos, added to the liquid media are beneficial, partially, it

is thought, because of the mechanical shielding of the bacteria from dissolved oxygen (Breden and Buswell, 1933). Reducing agents may include sodium sulfide, sodium hydrosulfite, or cysteine. Myelroie and Hungate (1954) described the use of hydrogen and palladium chloride as an effective reducing system for the growth of Methanobacterium formicicum isolated from sludge. The effectiveness of this reducing system supports the conclusion that a low oxidation-reduction potential in the medium is more important than the presence of a specific concentration of a reduced sulfur compound per se.

Substrates utilized by the methane bacteria as a group are relatively simple organic and inorganic compounds. The more usual substrates decomposed by bacteria, such as amino acids and carbohydrates, are not attacked by pure or nearly pure cultures of methane bacteria. Each separate species' substrate requirements are even more specific. For example, Methanobacterium formicicum oxidizes only hydrogen, carbon monoxide, and formate (Schnellen, 1947) and Methanobacillus omelianskii decomposes primary and secondary short-chain aliphatic alcohols (Barker, 1941) and hydrogen (Barker, 1943). When more complex substrates are oxidized, more than one species may have to be present, as with a valerate substrate where no less than three species are necessary for its decomposition. The cultures, thus once obtained, are maintained indefinitely since the major products of fermentation are gases which escape from the media and do not form toxic by-products.

Bacterial Methanogenesis

Prior to 1936, studies on the methane fermentations were done entirely on enrichment cultures containing a mixture of organisms which were fed a

single substrate. Even now, few investigations are carried on with pure cultures; highly enriched cultures are generally used. Since this thesis is primarily concerned with the utilization of one-carbon compounds, specifically formate, discussion will be limited to this particular group.

Söhngen (1910) was one of the first workers to detect the production of methane from a one-carbon compound. Using a crude culture of methane bacteria, he determined that a variety of fatty acids, including the calcium salt of formic acid, were converted quantitatively to carbon dioxide and methane. The same culture was shown to reduce carbon dioxide to methane.

Stephenson and Stickland (1933), using both pure and mixed cultures of methanogenic bacteria, hypothesized that the formation of methane is not caused by a direct reduction of formic acid; instead, the formic acid is first decomposed into hydrogen and carbon dioxide and then the carbon dioxide is reduced to methane by the excess hydrogen. The following overall reaction takes place in two separate stages:

$$4\ HCOOH \longrightarrow CH_4 + 3\ CO_2 + 2\ H_2O$$

$$HCOOH \longrightarrow H_2 + CO_2 \qquad (1)$$

$$4\ H_2 + CO_2 \longrightarrow CH_4 + H_2O \qquad (2)$$

The hypothesis was based on the observation that in the early stages of fermentation little methane was evident and quantities of carbon dioxide and hydrogen predominated, while later in the fermentation the amount of carbon dioxide and hydrogen diminished and the amount of methane increased. It is interesting to note that Stadtman and Barker (1951) assumed that only a small amount of methane would be evolved early in the methane fermentation since methane bacteria develop rather slowly. But when they analyzed 50 ml of gas

from a 250 ml enrichment culture which had been evolved in the first 18 hours of fermentation, nearly all the gas was methane. A small amount of carbon dioxide was present and no hydrogen was detectable.

Van Niel (Barker, 1956) suggested the carbon dioxide reduction theory for the origin of methane. He stated that organic compounds fermented by methane bacteria were oxidized to carbon dioxide. Then in the presence of suitable hydrogen donors, the carbon dioxide was reduced to methane.

Stadtman and Barker (1951) reported that the cell suspension of methanogenic bacteria they employed catalyzed a rapid exchange of carbons between carbon dioxide and formate. They suggested that this made carbon-14 impractical as a tracer, for within a few hours, both carbon dioxide and formic acid would be equally labeled. This was not found to be the case by Fina et al. (1960) who were able to use C^{14} in their work.

Several significant exceptions to the scheme of oxidation of organic compounds to carbon dioxide and subsequent reduction of the carbon dioxide to methane have been noted in the literature. Buswell and Sollo (1948), using an acetate substrate and carbon-14-labeled carbon dioxide, were able to prove that only a very small part of the methane was derived from the carbon dioxide. Pine and Barker (1956), in experiments with deuterium-labeled acetate confirmed this completely. The entire methyl group of acetic acid was transferred, as an unchanged unit, to methane.

Pine and Vishniac (1957) also demonstrated that the methyl carbon of acetic acid and the methyl alcohol carbon are reduced directly to methane. Both of these carbons appear to follow the same pathway and have a common one-carbon intermediate, CH_3X, the "X" indicating some unknown carrier.

Pine (1958) reported that a limited intact incorporation of deuteroformate into methane occurs in the methane fermentation of formate by Methanobacillus omelianskii, not normally formate-utilizing, and that a one-carbon transfer system may be related to the methane fermentation. However, it wasn't believed that a direct involvement could be demonstrated because of the postulated exchanges between formic acid, carbon dioxide, and other not well-defined compounds.

Fina et al. (1960) felt that the formic acid dehydrogenation to carbon dioxide and the following reduction of carbon dioxide to methane, as well as the exchange phenomenon between carbon dioxide and formate, might not be completely correct.

> There is no apparent reason why methane-producing bacteria should first oxidize the carbon of formate completely to carbon dioxide and then subsequently reduce it completely to methane. It has been established that methyl alcohol and the methyl carbon of acetic acid are reduced directly to methane. One might expect the formate carbon could be reduced similarly.

These authors showed by the use of $C^{14}O_2$ that a substantial part of the methane results from direct reduction of the formate without the formate first being oxidized to carbon dioxide and then reduced to methane. And as previously stated, the exchange phenomena between the formate and the carbon dioxide's carbons was not evident. Only a very small amount of activity was first apparent in the formate after 12 hours. Even after 35 hours the activity remained quite low.

The specific reaction involved in methane formation from carbon dioxide, acetate, formate, or methanol is not known. The simplest mechanism proposed for the formation of methane is a stepwise reduction of one-carbon compounds starting with carbon dioxide which is successively reduced to formate,

formaldehyde, methanol, and methane (Stephenson and Stickland, 1933). Kluyver and Schnellen (1947) re-investigated the "fermentation" of one-carbon compounds in the presence of hydrogen. They reported that Methanobacterium omelianskii could not reduce any one-carbon compound except carbon dioxide; Methanosarcina barkerii which "ferments" methanol and carbon monoxide was unable to reduce formate or formaldehyde; Methanobacterium formicicum "ferments" both carbon monoxide and formate, and reduces carbon dioxide with hydrogen, but is unable to reduce formaldehyde or methanol. Therefore, it is almost certain that the intermediates in the methane fermentation are not one-carbon compounds successively reduced in a stepwise fashion. The intermediates are of special interest in this thesis because of their relation to an "aerobe" isolated from a stock formate culture.

Phenomenon of Aerobes Growing under Anaerobic Conditions in the Methane Fermentation

The earliest reference concerned with the phenomenon of "strict" aerobes occurring in the anaerobic methane fermentation is to be found in an unpublished portion of the doctoral thesis by Fina (1950), although Dr. H. O. Halvorson, in a personal communication with Fina, stated that he had also noted the same phenomenon. Fina states:

> After the culture was established and was producing quantitative yields of gas from benzoic acid for a period of several months, nonspore-forming, Gram negative pseudomonas-like rods were isolated. They were isolated on solid media similar in composition to the liquid medium in which they had been growing. The only difference was that they had been subjected to air..... No explanation can be offered. It is certain that under the conditions the cultures were maintained, no oxygen could have been introduced. The culture flasks were subjected to rigorous tests.

> A vacuum of 10^{-4} mm Hg pressure was drawn and the system was carefully tested with a Tesla coil for leaks. The benzoic feed, kept in a bottle stoppered with a serum rubber vaccine cap, was boiled at 100° C. for at least 10 minutes before feeding.

Fina and Siegel (1954) briefly mentioned that strict aerobes had been found under anaerobic conditions in the methane fermentation in an Atomic Energy Commission Bulletin Progress Report.

The growth of aerobes in mixed culture under conditions that are definitely anaerobic and under which only anaerobes grow well, has certainly been observed by others. For instance, Guynes and Bennett (1959) and Isenberg and Bennett (1959) described aerobic pseudomonads which exist with anaerobic sulfate reducers in cutting oils. They felt that the aerobes lowered the oxidation-potential in the oils, allowing the sulfate reducers to multiply.

MATERIALS AND METHODS

Source of Culture and Preparation of Experimental Cultures

A formate-utilizing enriched stock culture, already present in the laboratory, served as a source of methanogenic bacteria and as a source of aerobic bacteria. It was actively producing methane at the time of transfer to experimental cultures. The enriched stock culture was originally obtained from a crude culture of rumen fluid.

Ten ml of the enriched stock culture were transferred to an all-glass 70 ml fermenter, similar in design to those shown in Fig. 1, containing Barker's solutions A and B (Barker, 1936, 1937) which were modified according to Fina *et al.* (1960). The solutions were composed of the following:

Fig. 1. Six all-glass fermenters, designated 1, 2, 3, 4, 5, and 6 were used during this investigation; Nidus consisted of fine sand and glass beads or asbestos, manometer fluid was a saturated solution of lithium chloride, and medium was a mixture of Barker's A and B mineral salts.

Solution A

Calcium formate 0.95 N	7.0 ml
$K_2HPO_4 \cdot 3H_2O$	0.5 gm
NH_4Cl	1.0 gm
$Mg\ Cl_2$	0.1 gm
Tap water	1,000 ml

Solution B

Cysteine	1.0 gm
Na_2CO_3	5.0 gm
Tap water	100 ml

The purpose of the cysteine was to lower the oxidation-reduction potential of the medium, thus establishing reducing conditions in the medium. The initial pH of 7.4 was adjusted to 6.8 with 0.1 N HCl. Shredded acid-washed asbestos was added as a nidus on which the bacteria could rest (Barker, 1936) and as an oxygen shield (Breden and Buswell, 1933). Gas produced was collected over a saturated solution of lithium chloride, used because very little carbon dioxide dissolved in it (Boell et al., 1939). Formate feeds were administered through a serum rubber stopper attached to a stopcock which had been fused to the glass fermenter in order to assure anaerobic conditions.

One millimole of formic acid[1], the sole source of carbon and energy, was administered each day for three days until the gas production had stabilized. Feed was increased to 2mM of formic acid a day until it was certain that the culture was in carbon balance (Table 1). Carbon balance here means that the amount of carbon fed into the system was returned in the gas yields each day.

1 One mM of formic acid yields one millimole of gas. This is about 23.5 ml of gas at 37°C. and at an altitude of 1,000 feet above sea level.

Table 1. Results of the fermenter's gas yield which shows it to be in carbon balance.

Days	Feeds: Amount of Formic Acid Substrate (mM)	Feeds: Theoretical Equivalent (ml)	Daily Yield (ml)	Total Unreturned Gas (ml)	Total Yield (ml)	Percent Carbon Recovery
0	1	0	0	0	0	0
1	1	25.3	18	7.3	18	71.1
2	1	50.6	6	26.6	24	47.8
3	2	75.9	30	20.6	54	71.1
4	2	126.5	47	24.2	101	79.8
5	2	177.1	71	5.1	172	96.6
6	2	227.7	32	23.7	204	89.6
7	2	278.1	56	18.1	260	93.5
8	2	328.7	61	7.7	321	97.6

For two days before the administration of the carbon-14-labeled formate 1 mM of "cold" calcium formate was given to the culture to prepare it for the radioactive calcium formate. Prior to the administration of $Ca(HC^{14}OO)_2$, volatile acid and pH determinations were made to detect the presence of any excess backfeed and to assure that the carbonate level in the media was not too low due to excessive acidity. The final pH of the substrate was 7.2 and 0.00869 mEq/ml of volatile acids were determined. The culture was then considered to be ready for the radioactive calcium formate. The schedule for the feeding of $Ca(HC^{14}OO)_2$ was as follows:

Hours	mM $Ca(HC^{14}OO)_2$ [1]	mM $Ca(HCOO)_2$
0	1.0	0
26	1.0	0
39	0.5	0.7
50	0.5	0.7

[1] 1mM $Ca(HC^{14}OO)_2$ ⟷ 0.4 microcuries.

It should be noted that the last two feeds were diluted with "cold" calcium formate in hopes that the specific activity curves of the CH_4, CO_2 and formate would be more sharply defined.

Carbon Dioxide, Methane, and Volatile Organic Acid Analysis

The gas collected was analyzed for carbon dioxide and methane content using a Burrell gas analysis apparatus (DE LUX BUILD-UP Model JS) with ball and socket joints. Procedures used are described in the Burrell Manual for Gas Analysis, Seventh Edition (1951) and by Fina et al., (1960). Methane was converted to carbon dioxide and water by combustion in the Burrell catalytic methane electric heater and the carbon dioxide was absorbed with NaOH. The absorbed carbon dioxide samples were precipitated by formation of insoluble barium carbonate (Calvin et al., 1949). The precipitate was recovered by centrifugation and rinsed several times with 95% ethyl alcohol and further centrifugation. All of the precipitate obtained was rinsed onto tared planchets with 95% ethyl alcohol and was dried with a heat lamp.

Volatile acid determinations were made on the culture liquor with a micro steam distillation apparatus using the procedure and apparatus designed by Fina and Sincher (1959). The distillate, after titration, was also placed on tared planchets and dried with a heat lamp. The amount of carbon-14 in the samples was determined by using a windowless gas flow alpha, beta, gamma, proportional counter.

Isolation of Aerobes from the Methane Fermentation

Isolation of "strict" aerobes was attempted after it was established that the formate-utilizing stock culture was definitely producing methane and carbon dioxide (15 ml of carbon dioxide and 9 ml of methane from 26.9 ml of gas). Samples of culture fluid for initial isolation were removed using aseptic technique. This was done in the following manner:

1. The serum rubber stopper attached to the stopcock of the fermenter was soaked in 70% ethyl alcohol for a minimum of five minutes.

2. The fermentation flask was vigorously shaken just before the sample was withdrawn to make certain that aerobes which tend to reside on the nidus would be included.

3. A positive pressure was applied by raising the leveling bulb to assure that any gas leaks would be outward.

4. One ml of culture liquor, removed with a sterile siliconized syringe and needle, was placed in a thin pour plate of nutrient agar.

Two types of bacterial colonies developed within 48 hours, a very few sprawling, irregular colonies of a Gram positive spore-forming bacillus but, predominantly, smooth colonies of a Gram negative bacillus. A portion of a colony of the Gram negative bacilli was transferred onto nitrogen-free mannitol agar plates (Lord, 1959). After growth on nitrogen-free media, a colony was then transferred to a series of agar "deeps." The aerobes grew only on the surface of the agar and these, thus isolated, were employed in the subsequent experiments.

Agar Deep Slicing Technique

Pure cultures of bacteria from the nitrogen-free medium and the nutrient agar "deeps" were transferred again to deep nutrient agar "shake" tubes. Control tubes were inoculated with Sarcina lutea, a known strict aerobe, and Pseudomonas aeruginosa, a facultative anaerobe. The tubes were incubated for 48 hours at 37° C. Agar was then removed from the tubes, sliced aseptically at different depths, and cultivated.

During the removal of agar and the slicing procedure, extreme care was taken to prevent contamination. The tubes were scored with a triangular file as close to the butt as possible and soaked for several minutes in 70% ethanol. With a sharp rap of the file, the end of the tube was cleanly knocked off without any contact being made with the agar. The agar was pushed out of the tube by inserting the file in through the mouth of the tube against the cotton plug. The agar cylinder slipped easily into a sterile Petri plate. To keep the agar from sliding, the bottom of the plate was lined with filter paper. Slices approximately 1 cm thick were made with a sterile sharp scalpel which was soaked in 95% ethanol and flamed between each slicing.

As soon as the agar slices were cut, they were placed in 99 ml water blanks and warmed for five minutes in a 60° C water bath. Although agar dispersal was a problem which was never entirely solved, even with vigorous shaking enough bacteria were suspended to obtain significant results. One ml from these water blanks was transferred to sterile Petri plates and mixed with a thin layer of nutrient agar. The plates were incubated for 48 hours at 37° C and the resulting colonies were counted.

Preparation of Apparatus for Aerobic Growth and Methane Fermentation Control

Six all-glass fermenters (Fig. 1) were prepared as described by Fina et al. (1960), with the exception that a mixture of acid-washed fine sand and glass beads were used as a nidus in place of shredded asbestos. The dried sand and glass beads were added to each fermenter. They were then autoclaved for 20 minutes at 121°C.

While the flasks were still hot from the autoclave, 15 ml of sterile Barker's B solution were added aseptically to each fermenter. The pH of each flask was adjusted to 6.8 to 7.0 with 1N HCl by using a pH meter. Flask 6 was broken during this procedure. Initial and final pH of the remaining five flasks after titration with 1N HCl was as follows:

Flask	Initial pH	Final pH
1	10.1	7.0
2	10.0	7.0
3	9.8	6.8
4	9.8	6.8
5	10.2	7.0

In order to drive off any dissolved oxygen, the flasks were reheated at 100° C in an Arnold steamer for ½ hour, after which the apparatus was immediately assembled. After assembly, the apparatus, while still hot, was well-rinsed with sterile nitrogen for five minutes. On analysis, the nitrogen gas showed less than 0.1% oxygen content. The inert gas was used to assure anaerobic conditions and to keep a slight positive pressure in the fermenters which eliminated the chance of oxygen entering the flasks. The completed sterile apparatus was allowed to stand overnight in order to detect changes in the manometer fluid levels.

The procedure of heating, rinsing the fermenters with nitrogen, and then allowing them to stand overnight was carried out for three days. Lithium chloride manometer settings remained stationary; therefore, it was certain that the seals of silicone grease on the glass joints and stopcocks were adequately air-tight.

The five fermenters were checked for sterility by making pour plates of one ml of medium removed aseptically from each fermenter. The plates were all negative after 48 hours. Each time a fermenter was entered, utmost care was taken to assure anaerobiosis and sterility. The fermenters were then inoculated.

One ml of an active gas-producing stock formate culture was added to fermenters designated 1, 2, and 3. From plate counts, it was determined that each ml contained 3.7×10^5 aerobes. Ten ml of a suspension of aerobes (1.43×10^6 bacteria/ml) were added to fermenters 4 and 5. An initial feed of 2 ml of formic acid was added to each fermenter. Plate counts and gas analysis were then initiated with fermenters 1, 2, and 3 every 6 hours for three days. Daily plate counts were started on fermenters 4 and 5. In each case, 1 ml of culture liquor was used.

During this entire series of experiments, fermenter 1 was treated as a normal fermentation and was considered to be the control flask. After the first three days, daily plate counts and gas analysis were done for 39 days and, thereafter, once a week for another month. Flask 1 was fed 2 mM of formic acid each day.

A steady state fermentation was established in flasks 2 and 3, during which time the aerobic population also became static. The aerobes were then eliminated from the fermentation by intermittent sterilization without harming

the methane bacteria. For four days the fermenters were placed in a 65° C incubator for a minimum of 2 hours. They were then allowed to incubate overnight at 37° C. For 7 days the fermentation was carried out minus the aerobic bacteria. After that time 1 ml of a suspension of aerobes (6.8×10^7 bacteria/m.) was injected into the fermenter. Each culture then was fed 2 mM of formic acid a day for 15 days and a normal fermentation was allowed to progress. Force-feeding of formic acid was begun then and carried out for 10 days, the feed being increased to 20 mM at 2 mM increments a day. Following the force feeding, at which time the fermenters were producing gas vigorously, the fermentation was arrested for 4 days by placing the flasks at room temperature. For the following two days fermenter 2 received 2 mM of formaldehyde a day and fermenter 3 received 2 mM of methanol a day. These fermenters were incubated, with no further feeds, for another two days. At this time the daily analyses were terminated and, following a feed of 5 mM of formic acid, each flask was rinsed with oxygen 3 times. Plate counts were continued weekly for a month.

Fermenters 4 and 5 were treated much differently. After 10 ml of the aerobe suspension was added, daily plate counts were done for 24 days. On the 24th day, 50 ml of sterile culture liquor from flask 1 was added to each of the two fermenters along with 1.5 ml of Barker's B solution which was destroyed in the sterilization process. Plate counts were continued for 10 days more. Then the same procedure was carried out with flasks 4 and 5 as with flasks 2 and 3.

Plate counts were done using 1 ml of culture liquor. Each time a sample was drawn, the contents of the fermenters were well-mixed by vigorous shaking. The samples were drawn with sterile siliconized syringes and 8-inch 21-gauge

needles. The fine sand seemed superior to asbestos where drawing of the samples was concerned as it did not tend to clog the needle.

There was a problem of colonies growing between the interface of the glass and the agar. The colonies would spread and thus make accurate counts difficult to achieve. Addition of chloral hydrate, as is used in isolation of Proteus sp., did not seem to help. It was, however, overcome by warming the plates slightly on a hot plate before adding the nutrient agar. The sample was added last. After 48 hours at 37° C, counts were made with the aid of the Quebec colony counter.

Identification of Aerobes

Determinative procedures in the identification of aerobic bacteria were employed in accordance with Bergey's Manual of Determinative Bacteriology, Seventh Edition (Breed et al., 1957) and Determinative Bacteriology Laboratory Manual (Lord, 1959).

Oxygen Uptake by Aerobes

Determination of oxygen uptake and, consequently substrate utilization, was carried out with Warburg constant volume respirometer (Umbreit, et al., 1949). Manometer fluid used was Brodies' solution. The cells, themselves, were washed in a mineral salts solution (Harris, 1961) which was later used as a cell diluent in the side-arm flasks.

EXPERIMENTAL RESULTS AND DISCUSSION

Reduction of Formic Acid to Methane

The results of this experiment, designed to show that a typical methane fermentation was used in this investigation, are presented in tabular form

in Table 2. In Plate I the specific activities are plotted graphically with time, time and specific activities being the coordinates. It can be seen that the specific activity curves for the methane and carbon dioxide are nearly parallel and that the formate specific activity curve intersects the methane and the carbon dioxide curves at their peaks.

Table 2. Specific activity of carbon dioxide, methane, and formate.

No. hrs.	Methane		Carbon dioxide		Formate	
	mM	Sp. Act.[1]	mM	Sp. Act.	mM	Sp. Act.
24	0.503	27,000	0.607	39,000	0.0236	78,700
34	0.316	60,800	0.377	66,800	0.00685	92,000
50	0.630	68,100	0.705	76,300	0.339	12,400
58	0.777	36,100	0.541	43,300	--	--

[1] Specific Activity (Sp. Act.): counts/minute/millimole.

An experiment similar to the one above was performed previously in this laboratory by Fina and Spence.[1] The methane and carbon dioxide curves plotted from their data, were also nearly parallel. The peaks of the curves were elongated because the specific activity dropped off much more slowly. This was corrected in the present experiment by changing the mode of feeding, that is, diluting the last two radioactive calcium formate feeds with cold calcium formate. Specific activity of the volatile acid distillates, not

[1] Personal communication.

EXPLANATION OF PLATE I

Graph of the specific activities of formate, carbon dioxide, and methane. Schedule for the feeding of radioactive calcium formate was as follows:

Hours	mM $Ca(HC^{14}OO)_2$	mM $Ca(HCOO)_2$
0	1.0	0
26	1.0	0
39	0.5	0.7
50	0.5	0.7

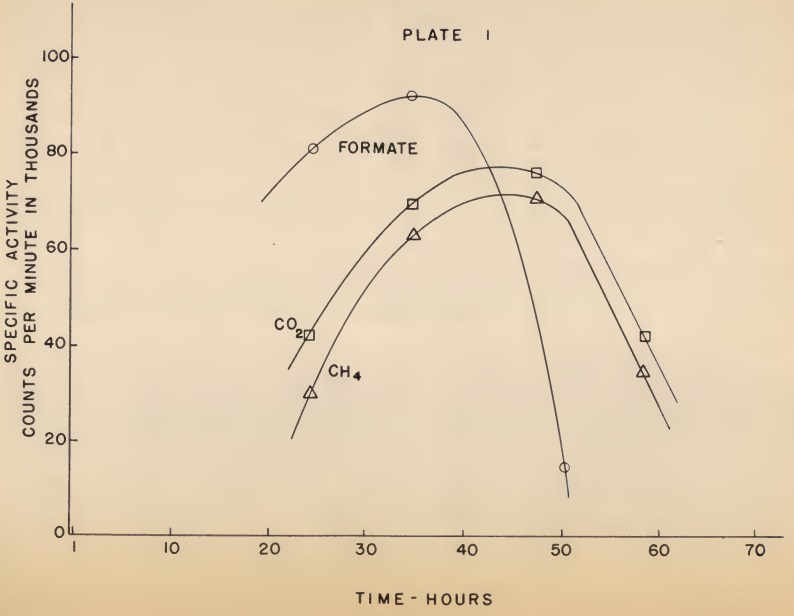


PLATE I
SPECIFIC ACTIVITY
COUNTS PER MINUTE IN THOUSANDS
100
80
60
40
20
0
FORMATE
CO_2
CH_4
1
10
20
30
40
50
60
70
TIME - HOURS

part of the data of Fina and Spence, were also included in this experiment to determine the activity of the formate.

Fina et al. (1960) attempted to show that methane could be formed by direct reduction of formate as well as by oxidation of formate to carbon dioxide and subsequent reduction of the carbon dioxide to methane. They labeled the carbon dioxide carbon, however, rather than the formate carbon with carbon-14 as was done in the present study. The same conclusion, however, could be drawn from both experiments.

According to the postulates of Zilversmit (Sacks, 1953) concerning the relation between specific activities of the precursor and the product in a biological system in which the initial activity of the tissue is zero, the carbon dioxide cannot be considered to be the sole precursor of methane. For carbon dioxide to meet the necessary conditions to be the sole precursor of methane, the following conditions would have to be satisfied:

1. The specific activity of carbon dioxide would have to be higher initially than the specific activity of methane.
2. The specific activity of carbon dioxide would have to reach its maximum before the specific activity of methane reaches its maximum.
3. When the specific activity of methane is maximum, the specific activity of carbon dioxide would have to be equal to the specific activity of methane.
4. The specific activity of methane would have to remain higher than that of carbon dioxide after the maximum specific activity of methane has been passed.

It can be seen from Plate I that the only condition met was the first. The specific activity of carbon dioxide was higher initially than that of methane. In view of the parallel methane and carbon dioxide curves, it is felt that this is not significant. The specific activities of carbon dioxide and methane reached their maximum peaks at the same time, but were equal at no time. The specific activity of methane was never higher than that of carbon dioxide. It is obvious that in this case, carbon dioxide was not the sole precursor of methane as conditions 2, 3, and 4 were not met. Had they been met, the methane curve would have intersected the carbon dioxide curve at its peak and remained higher than the carbon dioxide curve. This confirms data accumulated previously by Fina and Spence[1] (1960) and by Fina et al. (1960).

The formate carbon was labeled with C^{14} in this experiment to determine the actual fate of the carbon. Plate I shows that the specific activity curve of formate intersects the curves of both methane and carbon dioxide at their peaks of maximum specific activity. It then remains lower than the specific activities of carbon dioxide and methane. This suggests that methane is not derived entirely from the direct reduction of carbon dioxide. The relationship between the formate curve and/or methane curve indicates that these two gases are formed directly from formate and are independent of each other.

The original hypothesis of formate oxidation to carbon dioxide and subsequent reduction to methane was based on the gas evolution which occurred at different times during the experiment, carbon dioxide being evolved in greater quantities in the first 18 hours of the experiment (Stephenson and Stickland, 1933), rather than on tracer studies. The data by Pine (1958) suggested that

formate was reduced directly to methane, but he explained this on the basis of a rapid exchange between formate and carbon dioxide. The rapid exchange phenomenon was not noted by Fina et al. (1960) or by this author. It appears then that the pathway of methane formation from formate, according to this study, follows that postulated by Fina et al. (1960) and that of the methane formation from methyl alcohol and the methyl carbon of acetate (Buswell and Sollo, 1948; Pine and Barker, 1956; and Pine and Vishniac, 1957).

This investigation was attempted in order to establish that a typical methane fermentation was being used in the experiments which follow. It was felt that the data from the present study should confirm data from a previous study, which this does. The value of the experiment itself is not of primary importance. Other confirmatory evidence is noted in data accumulated and discussed later in this thesis.

Presence of Aerobes in the Methane Fermentation

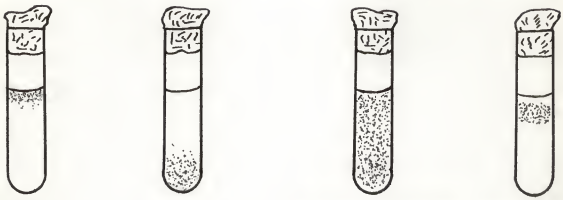
The detection of aerobes growing anaerobically in the methane fermentation was approached in two ways. First it was necessary to determine that the bacteria under investigation were strict aerobes. This was done by determining the depths to which they would grow in nutrient agar deeps and the comparing the results with a known aerobe, Sarcina lutea, and a facultative anaerobe, Pseudomonas aeruginosa. Results in Table 3 show that the isolated aerobe grew luxuriantly to a depth of 1 cm, Sarcina lutea colonies were too numerous to count at a depth of 2 cm, and Pseudomonas aeruginosa grew throughout the tube. As was mentioned earlier, these counts are merely indicative of the populations because of the difficulty with dispersal of the agar.

Table 3. Representative numbers of bacterial colonies from agar deep slices.

Depth of Agar Slice	Tube Number 2	3	5	6	*Sarcina lutea*	*Pseudomonas aeruginosa*
0.5 cm	TNTC[1]	TNTC	TNTC	TNTC	TNTC	TNTC
1.0 cm	TNTC	56	TNTC	TNTC	TNTC	TNTC
2.0 cm	36	3	248	3	TNTC	TNTC
3.0 cm	0	5	1	TNTC	46	TNTC
4.0 cm	0	2	4	4	1	TNTC
5.0 cm	8	TNTC	4	1	2	TNTC

[1] Too numerous to count (TNTC): more than 300 colonies in an agar plate of 10^7 dilution.

According to the classical definition of an obligate aerobe, molecular oxygen must be the ultimate hydrogen acceptor (Carpenter, 1961) or oxidizing agent (Stanier *et al.*, 1957). When this is the case, the bacteria tend to grow only at the very surface of agar deep tubes. In the standard microbiology text by Carpenter (1961), the oxygen relationships are illustrated as follows:



Aerobe Anaerobe Facultative anaerobe Microaerophile

It can be seen from Table 3 that the aerobe isolated from the methane fermentation, Sarcina lutea, and Pseudomonas aeruginosa grew in the following manner in nutrient agar:

Aerobe isolated from the methane fermentation

Sarcina lutea

Pseudomonas aeruginosa

The evidence, thus illustrated, leads one to suspect that the aerobe is obligately aerobic, but can multiply or at least exist for long periods of time in the strictly anaerobic methane fermentation.

The second approach was to study the aerobes in the methane fermentation itself under an inert gas atmosphere such as molecular nitrogen. In three flasks, a normal methane fermentation was established (Table 4) in which gas was produced in 36 hours. The gas which was produced after 36 hours and for the next eighteen hours was predominantly carbon dioxide. This essentially agrees with the data of Stephenson and Stickland (1933) who recovered carbon dioxide initially from their fermentations. Stadtman and Barker (1951), for some unexplained reason, found mainly methane in their cultures in the first eighteen hours of fermentation. After 60 hours had lapsed, the fermenters were producing gas at the accepted ratio of approximately 3 moles of carbon dioxide to 1 mole of methane (Stephenson and Stickland, 1933).

Table 4. Results of gas analysis during the first 78 hours using fermenters 1, 2, and 3. Each fermenter was fed 2 mM of formic acid. The percentages tabulated below were calculated from analysis of 20 ml aliquots of gas.

Hours	Fermenters 1			2			3		
	% CO_2	% CH_4	% Other	% CO_2	% CH_4	% Other	% CO_2	% CH_4	% Other
0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0
42	64	0	36	57	0	43	59	0	41
48	97	0	3	96	0	4	98	0	2
54	88	7	5	93	1	6	85	8	7
60	64	30	6	58	31	11	62	29	9
66	67	31	2	75	22	3	71	26	3
72	75	22	3	73	24	3	73	20	7
78	71	25	4	68	31	1	64	35	1

Data in Tables 5, 6, and 7 indicate the per cent of carbon recovery, showing that the three fermenters were in carbon balance throughout the experimentation period. In Table 8 the percentages of methane, carbon dioxide, and other unanalyzed gases are tabulated. It can be seen that an approximately 3 to 1 ratio of moles of carbon dioxide to methane remained constant during the first 38 days; therefore, the three flasks were considered to be typical of the methane fermentation in carbon balance.

While a steady state fermentation was being established in flasks 1, 2, and 3, the aerobe population also became stabilized (Table 9). In the first 60 hours of fermentation, the number of aerobes remained relatively low (Plates II, III, and IV). Then after 60 hours when carbon dioxide and methane began to be produced in the expected ratios, the aerobe population increased tenfold which indicates that the aerobes may be dependent on the methane fermentation itself and that an active methane fermentation is necessary in order for this organism to exist in an anaerobic situation.

A methane fermentation arrangement of apparatus and media was prepared in flasks 4 and 5 but they were inoculated only with a pure culture of the aerobes. No methane bacteria were present at all. Utmost care was taken to see that neither oxygen nor a contaminating organism were allowed to enter the flasks. After an initial feed of 5 mM of formic acid for each fermenter, daily plate counts (Table 10, Plates V and VI) were continued for 24 days. An additional feed of 5 mM of formic acid was added to each fermenter on the twelfth day. For the first 5 days the populations did not show a significant increase, but tended to remain static except for the second day when flask 4 showed a slight decrease. From the sixth to the ninth day, the numbers

Table 5. Data showing that Flask 1 was in carbon balance and produced gas in a typical fashion.

Days	Cumulative Feeds: Amount of Formic Acid Substrate (mM)	Cumulative Feeds: Theoretical Equivalent (ml)	Daily Yield (ml)	Total Unreturned Gas (ml)	Total Yield (ml)	Percent of Carbon Recovery
1	2	0	0	0	0	0
2	4	50.6	0	50.6	0	0
3	6	101.2	0	101.2	0	0
4	8	151.8	73	78.8	73	48.1
5	10	202.4	115	14.4	188	92.8
6	12	253.0	58	7.0	246	97.2
7	14	303.6	48	9.6	294	96.8
8	16	354.2	48	12.2	342	96.5
9	18	404.8	46	16.8	388	95.8
10	20	455.4	49	18.4	437	95.8
11	22	506.0	48	21.0	485	95.8
12	24	556.6	47	24.6	532	95.5
13	26	607.2	46	29.2	578	95.2
14	28	657.8	48	31.8	626	95.1
15	30	708.4	50	32.4	676	95.1
16	32	759.0	53	30.0	729	96.4
17	34	809.6	44	36.6	773	95.5
18	36	860.2	48	39.2	821	95.4
19	38	910.8	45	44.8	866	95.1
20	40	961.4	49	46.4	915	95.6
21	42	1012.0	42	55.0	957	94.5
22	44	1062.6	49	56.6	1006	94.2
23	46	1112.2	53	53.2	1059	95.2
24	48	1162.8	50	53.8	1109	95.4
25	50	1213.4	48	56.4	1157	95.4
26	52	1264.0	46	61.0	1203	94.4
27	54	1314.6	46	65.6	1249	95.2
28	56	1365.2	48	68.2	1297	95.7
29	58	1415.8	47	71.8	1344	95.6
30	60	1466.4	48	74.4	1392	94.2
31	62	1517.0	50	75.0	1442	95.2
32	64	1567.6	51	74.6	1493	95.2
33	66	1618.2	48	77.2	1541	95.3
34	68	1668.8	47	80.8	1588	95.1
35	70	1719.4	49	82.4	1637	95.2
36	72	1770.0	48	85.0	1685	94.6
37	74	1820.6	50	85.6	1735	95.2
38	76	1871.2	44	92.2	1779	95.2

Table 5. (concl.)

Days	Cumulative Feeds: Amount of Formic Acid Substrate (mM)	Cumulative Feeds: Theoretical Equivalent (ml)	Daily Yield (ml)	Total Unreturned Gas (ml)	Total Yield (ml)	Percent of Carbon Recovery
39	78	1921.8	51	91.8	1830	95.4
40	80	1992.4	47	95.4	1877	95.2
41	82	2023.0	48	98.0	1925	95.1
42	84	2073.6	48	100.6	1973	95.1
43	86	2124.2	51	100.2	2024	95.3
44	88	2174.8	52	98.8	2076	95.4
45	90	2225.4	50	99.4	2126	95.6
46	92	2276.0	49	101.0	2175	95.6
47	94	2326.6	48	103.6	2223	95.1
48	96	2377.2	50	104.2	2273	95.9

Table 6. Data showing gas yields of Flask 2.

Days	Cumulative Feeds: Amount of Formic Acid Substrate (mM)	Cumulative Feeds: Theoretical Equivalent (ml)	Daily Yield (ml)	Total Unreturned Gas (ml)	Total Yield (ml)	Percent of Carbon Recovery
1	2	0	0	0	0	0
2	4	50.6	0	0	0	0
3	6	101.2	26	75.2	26	25.7
4	8	151.8	76	75.8	108	62.5
5	10	202.4	93	1.4	201	99.3
6	12	253.0	47	5.0	248	98.0
7	14	303.6	45	10.6	293	96.5
8	16	354.2	47	14.2	340	96.3
9	18	404.8	49	15.8	389	96.1
10	20	455.4	47	19.4	436	95.7
11	22	506.0	44	26.0	480	94.9
12	24	555.6	48	27.6	528	95.1
13	26	607.2	54	25.2	582	95.8
14	28	657.8	49	26.8	631	95.8
15	30	708.4	47	30.4	678	95.6
16	32	759.0	50	31.0	728	95.9
17	34	809.6	52	29.6	780	97.5
18	36	860.2	51	29.2	831	96.5
19	38	910.8	48	31.8	879	96.5
20	40	961.4	52	30.4	931	96.8
21	42	1012.0	51	30.0	982	97.0
22	44	1062.6	54	22.6	1036	97.3
23	46	1112.2	47	29.2	1083	97.4
24	48	1162.8	48	31.8	1131	97.3
25	50	1213.4	46	36.4	1177	96.2
26	52	1264.0	48	39.0	1225	96.9
27	54	1314.6	54	35,6	1279	97.3
28	54	1314.6	6	29.6	1285	97.7
29	58	1314.6	2	27.6	1287	97.8
30	64	1415.8	102	26.8	1389	98.1
31	72	1567.6	148	30.6	1537	97.6
32	82	1770.0	220	13.0	1737	98.6
33	94	2023.0	252	14.0	2009	99.3
34	108	2326.6	297	20.6	2306	99.1
35	124	2680.8	349	25.8	2655	99.0
36	142	3085.6	396	34.6	3051	98.8
37	162	3541.0	444	46.0	3495	98.6
38	162	4047.0	494	58.0	3989	98.4
39	162	4047.0	12	46.0	4001	98.8
40	162	4047.0	4	42.0	4005	98.9
41	162	4047.0	1	39.0	4006	98.9
42	162	4047.0	0	39.0	4006	98.9

Table 7. Data showing gas yields of Flask 3.

Days	Cumulative Feeds: Amount of Formic Acid Substrate (mM)	Cumulative Feeds: Theoretical Equivalent (ml)	Daily Yield (ml)	Total Unreturned Gas (ml)	Total Yield (ml)	Percent of Carbon Recovery
1	2	0	0	0	0	0
2	4	50.6	0	0	0	0
3	6	101.2	51	50.2	51	50.4
4	8	151.8	92	7.8	143	94.8
5	10	202.4	48	7.4	191	94.3
6	12	253.0	46	16.0	237	93.7
7	14	303.6	51	15.6	288	94.8
8	16	354.2	46	20.2	334	96.2
9	18	404.8	52	18.8	386	95.3
10	20	455.4	46	23.4	432	94.9
11	22	506.0	42	32.2	474	93.6
12	24	555.6	45	36.6	519	93.4
13	26	607.2	49	39.2	568	93.5
14	28	657.8	49	40.8	617	93.8
15	30	708.4	48	43.4	665	93.8
16	32	759.0	49	45.0	714	94.1
17	34	809.6	50	45.6	764	94.3
18	36	860.2	52	44.2	816	94.8
19	38	910.8	46	48.4	862	94.6
20	40	961.4	52	47.4	914	95.1
21	42	1012.0	50	48.0	964	95.2
22	44	1067.6	56	42.6	1020	95.9
23	46	1112.2	52	40.2	1072	96.4
24	48	1162.8	48	42.8	1120	96.3
25	50	1213.4	46	47.4	1166	96.1
26	52	1264.0	50	48.0	1216	96.2
27	54	1314.6	54	44.6	1270	96.5
28	54	1314.6	8	36.6	1278	96.4
29	58	1314.6	0	36.6	1278	96.4
30	64	1415.8	102	35.8	1380	97.4
31	72	1567.6	146	41.6	1526	97.3
32	82	1770.0	206	38.0	1732	97.3
33	94	2023.0	256	35.0	1988	98.2
34	108	2326.6	298	40.6	2286	98.2
35	124	2680.8	349	45.8	2635	98.2
36	142	3085.6	395	55.6	3030	98.1
37	162	3541.0	446	65.0	3476	98.1
38	162	4047.0	506	65.0	3982	98.3
39	162	4047.0	12	53.0	3994	98.6
40	162	4047.0	2	51.0	3996	98.7
41	162	4047.0	0	51.0	3996	98.7
42	162	4047.0	0	51.0	3996	98.7

Table 8. Results of gas analysis after the first 72 hours. The percentages tabulated below were calculated from analysis of 20 ml aliquots of gas.

Days	Fermenters 1			2			3		
	% CO_2	% CH_4	% Other	% CO_2	% CH_4	% Other	% CO_2	% CH_4	% Other
4	71	26	3	69	30	1	72	27	1
5	73	25	2	69	25	2	69	22	9
6	69	28	3	70	29	1	68	26	6
7	67	29	4	69	28	3	68	27	5
8	69	30	1	66	33	1	69	31	0
9	73	26	1	74	25	1	73	25	2
10	74	26	0	73	24	3	75	23	2
11	74	26	0	72	26	2	73	26	1
12	73	26	1	76	23	1	73	24	3
13	74	25	1	72	26	2	72	26	2
14	72	25	3	73	25	2	74	24	2
15	74	23	3	75	24	1	76	24	0
16	74	25	1	75	23	2	74	24	2
17	76	23	1	72	24	4	74	25	1
18	74	24	2	75	22	3	69	28	3
19	71	22	7	72	24	6	74	25	1
20	74	24	2	74	23	3	73	26	1
21	75	24	1	73	24	3	73	23	4
22	73	24	3	72	24	4	73	25	2
23	74	24	2	71	25	4	71	25	4
24	69	25	6	71	25	4	73	25	2
25	74	26	0	73	24	3	71	26	3
26	72	24	4	74	23	3	73	24	3
27	69	24	7	71	26	3	73	24	3
28	70	24	6	72	24	4	74	23	3
29	70	26	4	69	24	7	66	29	5
30	72	27	1	73	25	2	73	25	2
31	73	24	3	71	24	5	71	23	6
32	74	24	2	73	23	6	73	24	3
33	74	24	2	73	23	4	73	24	3
34	73	24	3	74	23	3	72	24	4
35	74	23	3	73	24	3	73	24	3
36	72	23	5	74	24	2	73	24	3
37	74	24	2	73	24	3	73	23	4
38	71	25	4	72	24	4	72	24	4

Table 9. Plate counts of aerobes during the first 78 hours from flasks 1, 2, and 3. The inoculum from the stock formate culture contained 3.7×10^6 aerobes/ml.

Hours	Aerobe Plate Counts		
	Flask 1	Flask 2	Flask 3
0	700	2,100	800
6	1,800	2,200	2,900
12	1,200	3,300	2,600
18	1,500	2,800	1,600
24	3,100	3,700	2,200
30	3,800	4,200	3,500
36	6,100	8,300	9,400
42	14,400	11,500	13,200
48	20,200	27,300	39,100
54	29,000	570,000	460,000
60	254,000	320,000	470,000
66	220,000	257,000	318,000
72	720,000	930,000	460,000
78	810,000	360,000	280,000

Table 10. Tabulation of daily plate counts from flasks 4 and 5. No methane bacteria were present in these flasks.

Days	aerobes/ml Flask 4	aerobes/ml Flask 5
1	3,200	2,900
2	1,200	3,900
3	3,600	2,500
4	3,900	3,100
5	4,100	6,700
6	580	620
7	120	280
8	20[1]	80
9	2	0
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16	0	0
17	0	0
18	2	0
19	1	0
20	0	0
21	1	0
22	0	1
23	3	1
24	0	0
25	8,300	5,100
26	4,700	6,400
27	1,640	1,210
28	920	1,270
29	63	55
30	6	7
31	0	1
32	0	0
33	0	0
34	0	0

[1] Plate counts made with 1 ml of undiluted culture liquor, having less than 30 colonies on them, can only be considered estimations.

EXPLANATION OF PLATE II

Graph of the log numbers of aerobic organisms of flask 1 during the first 72 hours of fermentation. Samples were drawn every 6 hours. Fermenters received 2 mM of formic acid every 24 hours.

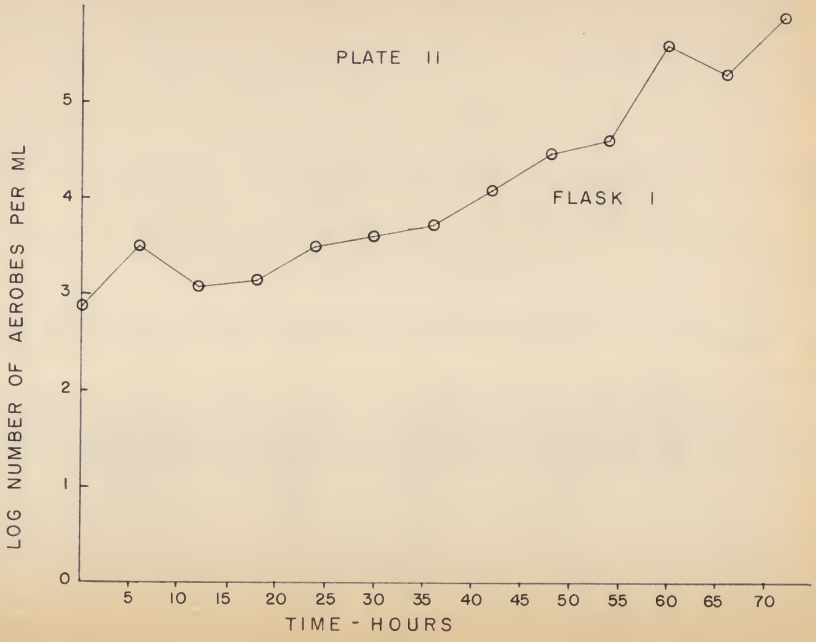


PLATE II
FLASK I
LOG NUMBER OF AEROBES PER ML
0
1
2
3
4
5
5
10
15
20
25
30
35
40
45
50
55
60
65
70
TIME - HOURS

EXPLANATION OF PLATE III

Graph of the log numbers of aerobic organisms of flask 2 during the first 72 hours of fermentation. Samples were drawn every 6 hours. Fermenters received 2 mM of formic acid every 24 hours.

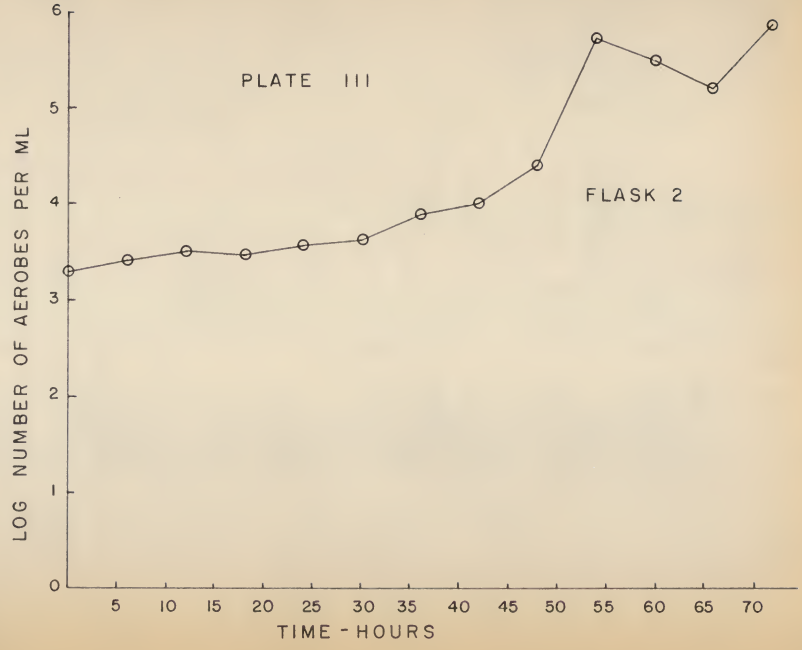


PLATE III
FLASK 2
LOG NUMBER OF AEROBES PER ML
0
1
2
3
4
5
6
5
10
15
20
25
30
35
40
45
50
55
60
65
70
TIME - HOURS

EXPLANATION OF PLATE IV

Graph of the log numbers of aerobic organisms of flask 3 during the first 72 hours of fermentation. Samples were drawn every 6 hours. Fermenters received 2 mM of formic acid every 24 hours.

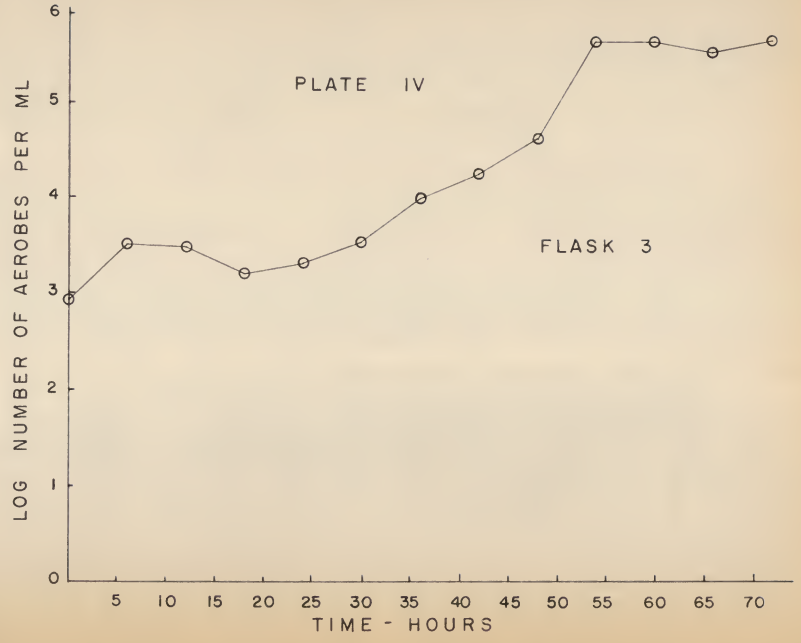


PLATE IV
FLASK 3
LOG NUMBER OF AEROBES PER ML
0
1
2
3
4
5
6
5
10
15
20
25
30
35
40
45
50
55
60
65
70
TIME - HOURS

EXPLANATION OF PLATE V

Graph of the log numbers of aerobic organisms in flask 5 in pure culture using the methane fermentation apparatus and media. Feeds of 5 mM of formic acid were added to the fermenter initially and after 12 days. On the 24th day, 50 ml of sterile medium was transferred to flask 5 from flask 1.

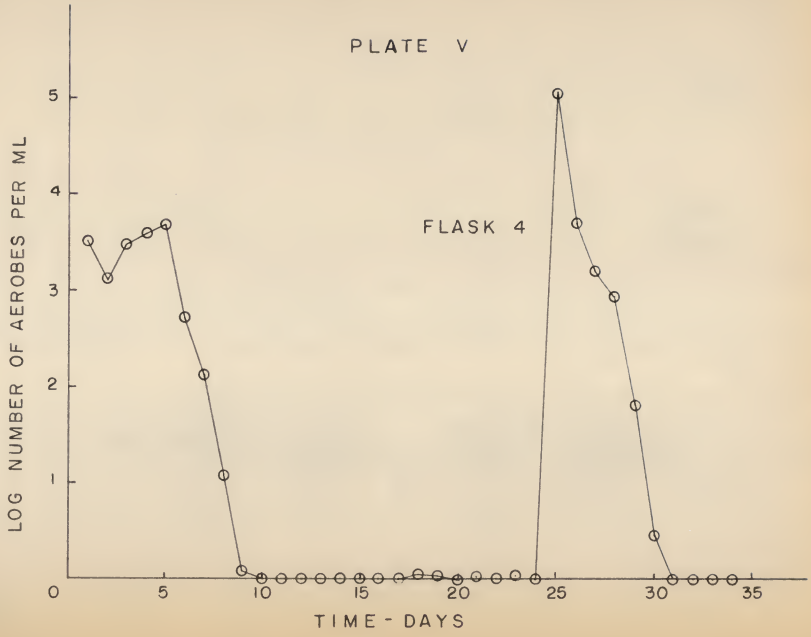


PLATE V
FLASK 4
LOG NUMBER OF AEROBES PER ML
TIME - DAYS
0
1
2
3
4
5
0
5
10
15
20
25
30
35

EXPLANATION OF PLATE VI

Graph of the log numbers of aerobic organisms in flask 5 in pure culture using the methane fermentation apparatus and media. Feeds of 5 mM of formic acid were added to the fermenter initially and after 12 days. On the 24th day, 50 ml of sterile medium was transferred to flask 6 from flask 1.


PLATE VI
FLASK 5
LOG NUMBER OF AEROBES PER ML
0
1
2
3
4
5
5
10
15
20
25
30
35
TIME - DAYS

decreased to a very low number per ml. Through the ninth to the 24th days, only an occasional colony was observed on the plates. The additional formic acid feed seemed to have no effect. On the 24th day, 50 ml of material from flask 1 were autoclaved and placed into fermenters 4 and 5. By the next day the populations had increased from less than 1 aerobe/ml to several thousand aerobes/ml at which level they remained for 3 days. From the peak at 25 days the aerobe count dwindled to almost zero in 31 days and remained at zero.

That the aerobe did not flourish anaerobically in the absence of the methane fermentation is significant. Apparently some intermediate connected with the methane fermentation itself is necessary for its growth, since the aerobe multiplied for a brief period of time when a sterile 50 ml portion from a normal fermentation was added. It is not thought that the increase was due to a leakage of air during the transfer or to dissolved oxygen in the sterile culture liquor. A positive gas pressure was maintained during the transfer and the liquor had been cooled rather rapidly for five minutes in the freezing compartment of a refrigerator before being added.

Since it is postulated in this thesis that an aerobe isolated in pure culture is dependent on an active methane fermentation for its maintenance, the question was raised as to whether the methane fermentation was dependent on the aerobe. Flask 1 continued as the control fermenter. In flasks 2 and 3, the aerobes were destroyed by intermittent sterilization and the methane fermentation minus the aerobes was continued for 7 days. It should be noted that the process of heating the flasks did not disturb the normal gas production. Also, it should be noted that amounts of gas were recorded before the fermenters were heated. In Table 11 the response of the aerobic population

to heating at 65° C for 2 hours is tabulated. Gas analysis and yields may be found in Tables 6, 7, and 8. From these tables it can be seen that the methane fermentation remained in a steady state while the aerobe population was destroyed; therefore, it can be stated that the methane fermentation apparently is not dependent upon the presence of aerobe population.

Table 11. Results of plate counts of aerobes/ml of culture fluid for flasks 1, 2, and 3. Flask 1 is the control fermenter. Flasks 2 and 3 were heated at 65°C for two hours during the first 4 days.

Days	Plate Counts (aerobes/ml)		
	Flask 1	Flask 2	Flask 3
1	87,000	950,000	810,000
2	12,600	15,700	12,200
3	13,500	6,200	4,100
4	11,600	1,200	1,800
5	13,400	10	0
6	15,000	0	0
7	12,200	0	0
8	10,400	0	0
9	14,900	0	0
10	21,100	0	0
11	13,400	0	0

The next phase of the study was concerned with disturbing the active methane fermentation. This was done in two different ways, first by increasing the gas production and then by arresting it. One ml of a suspension of aerobes (6.8×10^7/ml) was added back to each fermenter. Each culture was then fed 2 mM of formic acid a day for 15 days. On the fifteenth day, force-feeding of the fermentations was begun in order to determine any response the aerobes might make to the presence of a quite vigorous fermentation. For 10 days, the feeds were increased 2 mM a day up to 20 mM of formic acid. The fermenters during this increase remained in carbon balance (Tables 6 and 7). Plate count results of this phase of the investigation are in Table 12 and are plotted graphically in Plate VII.

Immediately after the reinoculation of flasks 2 and 3 with a suspension of aerobes, the population began to increase but became stable in three days. Then, during the process of force feeding, the numbers increased 1,000 times. It has been noted several times in this laboratory that when an active and steady state fermentation is disturbed, the number of aerobes increases. Whether this increase in population was a direct result of the force-feeding and the presence of a greater concentration of some unknown intermediate or whether it was a result of simply disturbing the fermentation in some way by the increase in formic acid is not known. However, when the fermentation is disturbed by exposure to a temperature lower than 37° C, a much greater increase in the population occurs.

For four days, fermenters 2 and 3 were removed from the 37° C incubator and incubated at room temperature, which in this case varied from 22° C to 27° C. The production of gas was arrested entirely (Table 9, 39th day) and the number of aerobes in flask 2 increased from 4.50×10^5/ml initially to

Table 12. Results of plate counts of aerobes/ml of culture fluid for flasks 1, 2, and 3. Flask 1 is the control fermenter. Flasks 2 and 3 were force-fed formic acid in 2 mM increments up to 20 mM after receiving only 2 mM/day for 15 days.

Days	Plate Counts (aerobes/ml)		
	Flask 1	Flask 2	Flask 3
1	15,200	470	320
2	14,000	830	610
3	15,100	4,200	3,700
4	19,200	8,100	4,700
5	12,400	3,400	5,800
6	14,300	5,500	5,200
7	22,200	6,200	6,900
8	15,700	3,700	6,100
9	16,500	6,900	9,300
10	13,100	4,800	10,100
11	13,700	5,100	6,600
12	17,100	3,500	6,200
13	15,300	4,400	3,400
14	16,400	7,500	5,100
15	16,100	3,800	3,700
16	13,100	8,200	6,200
17	12,900	45,000	32,000
18	15,300	67,000	32,000
19	14,700	103,000	126,000
20	14,300	212,000	298,000
21	11,000	310,000	370,000
22	11,600	550,000	1,710,000
23	19,100	700,000	890,000
24	13,000	360,000	800,000
25	12,200	450,000	590,000

EXPLANATION OF PLATE VII

Graph of the log numbers of aerobic organisms in flask 1, the control flask, during the experiment in which flasks 2 and 3 were force-fed formic acid. Flask 1 received 2 mM of formic acid a day.

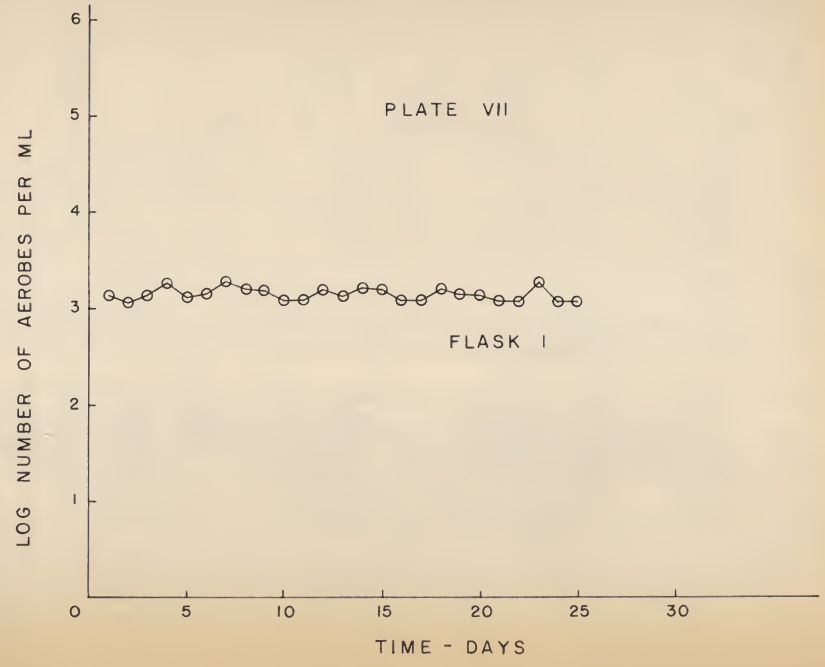


PLATE VII
LOG NUMBER OF AEROBES PER ML
6
5
4
3
2
1
0
5
10
15
20
25
30
TIME - DAYS
FLASK I

EXPLANATION OF PLATE VIII

Graph of the log numbers of aerobic organisms in flask 2. The first 15 days flask 2 was fed 2 mM of formic acid a day. The following 10 days the feed was increased 2 mM a day, the final feed being 20 mM of formic acid.

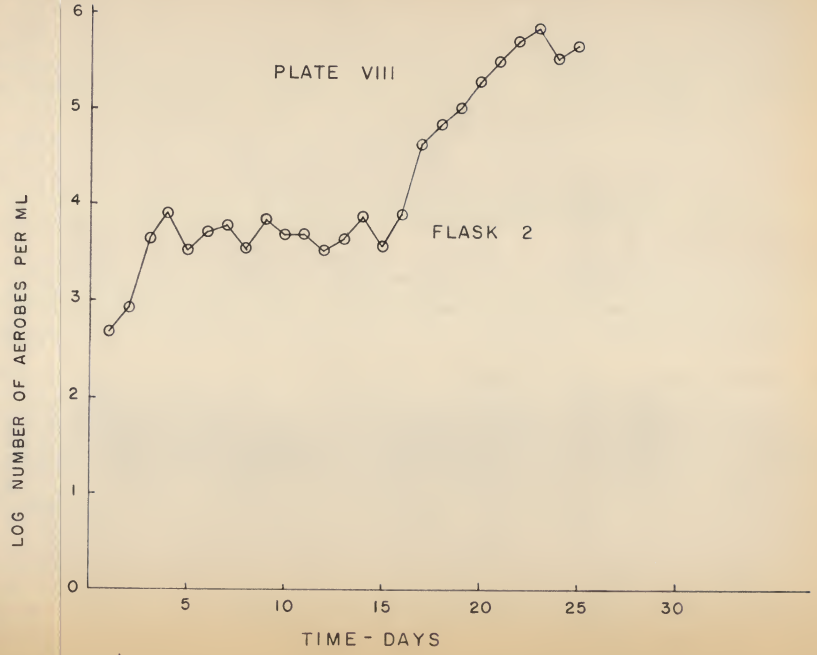


PLATE VIII
FLASK 2
LOG NUMBER OF AEROBES PER ML
TIME - DAYS
0
1
2
3
4
5
6
5
10
15
20
25
30

EXPLANATION OF PLATE IX

Graph of the log numbers of aerobic organisms in flask 3. The first 15 days flask 3 was fed 2 mM of formic acid a day. The following 10 days the feed was increased 2 mM a day, the final feed being 20 mM of formic acid.

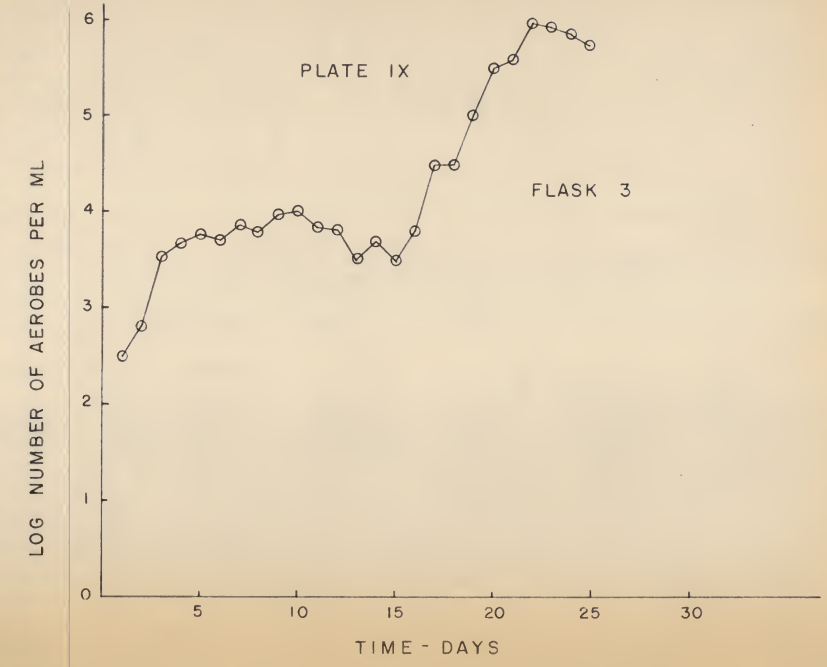


PLATE IX
FLASK 3
LOG NUMBER OF AEROBES PER ML
TIME - DAYS
0
1
2
3
4
5
6
5
10
15
20
25
30

1.02×10^7/ml in two days (Table 13). In flask 3 a similar increase occurred, 5.9×10^5/ml in two days. The increases were similar to the response obtained when the feeds were greatly increased. Therefore, it is felt that one may make the statement that an increase in populations occurred when the mode of caring for the fermenters was changed, but the exact reason is still obscure.

Table 13. Results of plate counts of aerobes/ml in flasks 1, 2, and 3. Flasks 2 and 3 were placed at room temperature for 4 days. Flask 1 remained at 37° C.

Days	Plate Counts (aerobes/ml)		
	Flask 1	Flask 2	Flask 3
Initial	12,200	450,000	590,000
1	18,400	4,600,000	6,700,000
2	16,500	10,200,000	15,600,000
3	17,200	2,610,000	2,020,000
4	14,900	680,000	560,000

In order to determine whether or not the aerobes could utilize some of the postulated intermediates in the methane fermentation, flask 2 was fed 2 mM of formaldehyde for 2 days and flask 3 was fed 2 mM of methanol for two days. The feeds were continued for only two days since, according to Stanier's postulated theory of simultaneous adaptation (Clifton, 1957), these compounds, if they are intermediates in the methane fermentation, would be utilized immediately by the methane bacteria and perhaps by the aerobes. Feeds continued over a longer period of time might induce the ability of the bacteria to utilize them whether they are intermediates or not. It can be seen from Table 9

that no gas was produced from these substrates. However, the fermenters were fed these substrates immediately after they had been kept at room temperature for four days, so it is felt that no conclusion may be drawn from the lack of gas production. Of primary interest is the response of the aerobes. The populations remained stationary and there was no apparent increase (Table 14). Because of this it was felt that neither formaldehyde nor methanol were utilized by the aerobic bacteria.

Table 14. Results of plate counts of aerobes/ml in flasks 1, 2, and 3. Flask 2 was fed 2 mM of formaldehyde for 2 days, flask 3 was fed 2 mM of methanol for 2 days, and flask 1 was fed 2 mM of formic acid for 2 days.

Days	Plate Counts (aerobes/ml)		
	Flask 1	Flask 2	Flask 3
Initial	14,900	680,000	560,000
1	14,200	410,000	320,000
2	12,200	610,000	680,000
3	13,200	261,000	408,000
4	18,200	71,000	109,000

Utilization of formaldehyde, methanol, as well as glucose, calcium formate, sodium acetate, sodium succinate, sodium citrate, and cysteine in m/20 concentrations, was further determined by the conventional Warburg techniques; the temperature was 30° C, the gas phase was air, and the numbers of bacteria used were controlled by adjusting the bacterial suspensions to 3.3×10^4/ml to the same absorbency (Optical density 0.22 at 600 mu) with the Bausch and Lomb "Spectronic 20." Oxidation rates were corrected for endogenous oxygen uptake.

Results (Table 15) indicate that sodium acetate, sodium succinate, and sodium citrate were the only substrates used of those tested. Very little oxygen uptake was recorded with formaldehyde, methanol, glucose, calcium formate, and cysteine. It appears then that the oxidation of intermediates postulated for the methane fermentation is limited when the aerobes are concerned since after 60 minutes, the oxygen uptake with formaldehyde was only 19.06 microliters and with methanol was 20.97 microliters. Neither can it be assumed that calcium formate or cysteine, are useful substrates since the oxygen uptake in 60 minutes of each is 30.16 and 15.02 microliters, respectively. Although glucose was not utilized (7.47 microliters of O_2/hour), some of the intermediates of glucose metabolism were readily used, especially sodium citrate (174.14 microliters of O_2/hour). Sodium acetate (91.74 microliters of O_2/hour) and sodium succinate (74.27 microliters of O_2/hour) were also oxidized in significant amounts.

It seemed necessary, in this series of experiments, to determine the response of all five flasks to oxygen. Each fermenter was rinsed three times with oxygen, following which they were fed a final feed of 5 mM of formic acid. Plate counts were done weekly for one month. Gas production stopped entirely, since the methane bacteria are extremely sensitive to oxygen. Flasks 1, 2, 3, 4, and 5 showed much the same pattern, that is, an increase in organisms with the maximum numbers occurring after the second week. The populations decreased the third and fourth week undoubtedly because of a depletion of the substrate. It is obvious then that the "aerobes" tend to flourish more under aerobic conditions, but can manage to exist and sometimes multiply in the anaerobic methane fermentation.

Table 15. Measurement of oxidation rates of various substrates by an aerobe isolated from the methane fermentation.

Substrate	Respiration rates measured in microliters of O_2					
	15 min.	30 min.	45 min.	60 min.	75 min.	90 min.
Calcium formate	7.35	6.42	8.46	7.03	5.18	4.04
Cysteine	4.98	1.03	2.45	6.56	3.41	0
Formaldehyde	3.09	4.06	4.56	7.89	9.80	5.15
Glucose	1.61	2.06	1.12	2.69	1.58	1.78
Methanol	4.90	5.31	6.06	4.70	4.64	3.09
Sodium acetate	29.20	23.00	24.20	15.34	18.66	18.38
Sodium citrate	44.76	40.89	47.03	41.46	35.61	32.10
Sodium succinate	14.93	19.68	22.06	17.60	16.48	13.45

Table 16. Response of aerobic bacteria in the methane fermentation to the addition of oxygen to the fermentation flasks.

Week	Number of aerobes/ml in thousands				
	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5
0	31	261	38	1	0
1	168	2420	1980	2880	3030
2	5100	4340	3100	5900	1100
3	640	710	132	410	232
4	21	48	102	86	14

Determinative Procedures

Interest in the identity of the "aerobe" led to the use of the following determinative procedures, and the results. Bacteria used were isolated from the methane fermentation and showed aerobic growth in agar deeps. Culturally and biochemically similar organisms were isolated repeatedly from the fermenters. Characteristics are as follows:

Cell morphology on nutrient agar after 2 days at 37° C.

Shape of vegetative cells: bacillus (square ends).
Arrangement of cells: single.
Irregular forms: elongated and narrow on nutrient agar containing 0.5% lithium chloride.
Size of cells: 0.45 - 0.60 x 0.60 - 0.75 microns.
Motility in hanging drop: uncertain.
Motility in semi-solid agar: definite.
Flagella stain: polar, monotrichous flagella.
Endospores: none observed.
Heat test: negative.

Staining characteristics after 2 days' growth on nutrient agar at 37° C.

Gram stain: negative.
Acid fast stain: negative
Capsule stain: positive
Granule stain: negative.

Colonies on nutrient agar plates after 2 days at 37° C.

Form: circular, 1 to 3 mm in diameter.
Elevation: convex.
Surface: smooth.
Margin: entire.
Density: opaque.
Internal structure: finely granular.
Chromogenesis: cream-colored.
Texture: viscid, becoming extremely tenacious after a week.
Fluorescence: green with ultra violet light.

Colonies on nitrogen-free mannitol agar after 3 days at 37° C.

Form: punctiform, 1 mm in diameter.
Elevation: flat.
Surface: smooth.
Margin: erose.
Density: opaque.
Internal structure: granular.
Chromogenesis: white.
Texture: extremely tenacious, difficult to transfer.

Stroke on nutrient agar after 2 days at 37° C.

Amount of growth: abundant.
Form: filiform.
Texture: viscid.
Density: opaque.
Chromogenesis: cream-colored.
Luster: shiny.
Odor: none.

Relation to free oxygen: aerobic.

Catalase test: positive.

Temperature relationship:

28° C: moderate growth.
37° C: luxuriant growth.
45° C: no growth.

Biochemical reactions, 37° C.

The following carbohydrates showed no pH change after 10 days: arabinose, rhamnose, xylose, glucose, lactose, sucrose, maltose, trehalose, raffinose, inulin, dextrin, starch, glycerol, mannitol, sorbitol, inositol, dulcitol, salicin, cellobiose, mannose.

Litmus milk observations at 37° C showed the following reactions:

pH: no change in 10 days.
Acid curd: none in 10 days.
Rennet curd: none in 10 days.
Peptonization: none in 10 days.
Gas production: none in 10 days.
Litmus reduction: reduction occurred in 24 hours.

Starch hydrolysis: negative.
Casein hydrolysis: negative.
Gelatin hydrolysis, gelatin stab and Frazier's gelatin agar: negative.
KNO_3 reduced to KNO_2: positive, no gas formed.
Urea used as a nitrogen source: negative.
NH_3 used as a nitrogen source: positive.
H_2S produced in peptone iron agar: negative.
Methyl Red test: negative.
Indole production: negative.
Citrate utilization: positive.
Mucate utilization: negative.
Phenol Red Tartrate Agar: negative.
Naphthalene salts solution: no growth.
Phenol salts solution: no growth.
Cellulose salts: no growth.
5% NaCl broth: no growth.

Growth in nutrient broth: pellicle formed.

Pigment production:

Proteose peptone agar: pale yellow, water-soluble.
Gelatin: pale yellow, water-soluble.
Potato slants: no growth.
Sabouraud's agar: no growth.

Lord's carbon-free ammonium phosphate agar: no growth.

Nitrogen-free media:

Nitrogen-free mannitol agar: growth.
Heinnemann's glucose agar: growth.

According to Bergey's Manual of Determinative Bacteriology, Seventh Edition (1957), the aerobe isolated in pure culture probably belongs to the genus Pseudomonas; at least it is a pseudomonas-like organism. It is a Gram negative bacillus, motile by means of a polar, monotrichous flagellum. It develops a fluorescent, diffusible pale yellow pigment on gelatin media. Carbohydrates are not attacked fermentatively and nitrates are reduced to nitrate. Growth is evident on nitrogen-free media. Further procedures must be accomplished before a genus and, certainly, before a species name is assigned to this organism.

SUMMARY

The first phase of this thesis dealt with the study of the pathway of methane fermentation from a formate substrate in order to establish that the cultures used were normal and typical methane fermentations. Data accumulated showed that the pathway of formate utilization followed that elaborated by Fina et al. (1960) and gas production was similar to that determined by Stephenson and Stickland (1933).

The second phase of this investigation concerned an aerobic pseudomonas-like organism found in an established methane fermentation. The presence of aerobic bacteria flourishing under such strictly anaerobic conditions is difficult to accept at first glance, but it must be remembered that rarely in nature do bacteria exist in pure culture. Guynes and Bennett (1959) and Isenberg and Bennett (1959) found aerobes growing with anaerobic sulfate reducers in cutting

oils. They theorized that the aerobes lower the oxidation-reduction potential of the medium so anaerobes can grow. The aerobes and anaerobes in this case function together and are not independent of each other. In the present investigation, however, the culture liquor from the methane fermentation is highly reduced because of the presence of cysteine in the medium. Some other raison d'etre must eventually be elucidated.

The question arose as to whether the aerobe's anaerobic growth was dependent on the methane fermentation and, conversely, whether the success of the methane fermentation is dependent on the presence of the aerobe. It appears, from the data accumulated that the aerobe is present in very low numbers in an inactive methane culture. In a steady state fermentation, the aerobic population is static. When an active fermentation is disturbed in some way, either by increasing the rate of gas production or arresting it, the aerobe population increases rapidly. When the aerobe alone is added to the methane fermentation set-up, its population rapidly decreases to less than one aerobe per ml. Sterile material from an active gas producing culture transferred to such a fermenter, causes a substantial rise in the number of aerobes, making one suspect that the aerobe exists on an intermediate formed during formate reduction. However, neither the aerobe nor the methane bacteria responded to the addition of the postulated intermediates (formaldehyde and methanol) of the methane fermentation. Also oxygen uptake was extremely limited when the aerobe was exposed to methanol and formaldehyde substrate. The conclusion, then, may be drawn that the methane fermentation is not dependent on the aerobe, but the maintenance of the aerobe in the anaerobic methane fermentation is dependent on the presence of an active methane

fermentation and the growth of the aerobe is sharply increased when the fermentation is disturbed. When oxygen is added to the fermenters, the oxygen sensitive methane fermentation ceases and the aerobe's increase is immediate and rapid.

Since in nutrient agar deeps, the organism appears to be a strict aerobe, and has been found, thus far, to grow anaerobically only in this one situation, it is difficult to apply the term facultative anaerobic to it. Consequently, throughout this thesis it has been referred to as an aerobe.

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AEROBES ASSOCIATED WITH THE METHANE FERMENTATION DURING FORMATE UTILIZATION

by

MARILYN EMILE RINEHART

B. A., Kansas State Teachers College of Emporia, 1958

AN ABSTRACT OF A MASTER'S THESIS

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MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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A pseudomonas-like strictly aerobic bacterium was isolated from an active, gas-producing enriched methane fermentation which utilized a formate substrate. The strictly anaerobic, oxygen-sensitive methanogenic culture was found to be typical of a normal methane fermentation by determining ratios of gases produced and by following the fate of the formate carbon, labeled with carbon-14. The pathway of formate utilization agreed with the one published by Fina, et al. and the ratio of moles of carbon dioxide to moles of methane formed followed the accepted ratio of 3/1 established by Stephenson and Stickland.

In order to determine the relationship of the aerobe to the methane bacteria, a series of experiments were performed. Three gas-producing fermentations in carbon balance were developed, one of which was maintained per se as a control, receiving only feeds of 2 mM of formic acid a day. Aerobes transferred in the inoculum to the remaining two flasks were destroyed by intermittent sterilization. Gas production remained at the same levels without the aerobe, thus indicating that the methane fermentation is not dependent on the presence of the aerobe.

Another pair of fermentation flasks was prepared, only in this case, a pure culture of the aerobe was added; no methane bacteria were present. After nine days, less than one aerobe/ml of culture fluid remained. The aerobic count increased again on the addition of sterile material from an active gas-producing culture.

Without the methane fermentation, the aerobe has not yet been found to exist anaerobically. With an active methane fermentation in carbon balance, the aerobic population remains static. When an active fermentation is

disturbed, either by increasing gas-production or arresting it, the response of the aerobe is enhanced greatly. Gas-production was increased by increasing formic acid feeds from 2 mM to 20 mM/day using 2 mM increments each day. Gas production was arrested by lowering the temperature of incubation. Addition of oxygen to all of the fermenters resulted in an increase in the aerobe population.

It was thought that the aerobe might be utilizing an intermediate from the fermentation of formate. Methanol and formaldehyde have been postulated as intermediates in this fermentation by Stephenson and Stickland, but the author was unable to demonstrate that the aerobes utilized either formaldehyde or methanol by using them as substrates in the fermentation and in oxygen uptake determinations.

There is little doubt that the organism isolated from the strictly anaerobic methane fermentation is aerobic in nature. When cultivated in nutrient agar deep tubes, the aerobic organism was recovered in significant numbers only from a slice of the top 1 cm of the agar.